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PROTEIN INTERACTIONS WITH ORGANIC MOLECULES

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The results of earlier investigations of interactions of serum albumin with organic anions and cations are reviewed briefly and new studies with neutral molecules are described. Numerous observations indicate that this protein undergoes marked configurational changes as the pH of the environment is altered. New data are also presented for the interaction of caseins with anions. These results together with published observations on the effect of denaturation on binding are explained in terms of the content of polar residues in protein molecules.

In its most general sense, a description of protein interactions with organic molecules should include specific interactions such as between enzymes and substrates or between antibodies and haptens, as well as non-specific complexes of which those of serum albumin form the outstanding examples. This paper, however, will limit itself to some representative non-specific interactions and an attempt will be made to co-ordinate a variety of experimental facts in terms of a reasonable molecular picture.

From a molecular viewpoint, an explanation of non-specific interactions presents problems which in many respects are more difficult than those encountered with specific complexes. With the latter, the lock and key concept, suitably refined to include modern ideas on molecular structure, serves well to explain known phenomena as well as to suggest new promising metabolic inhibitors or competitors in serological reactions. A principle of complementarity in itself is inadequate, however, to account simultaneously for the unusual affinity of particular proteins for small molecules as well as for the remarkable absence of discrimination with respect to structure of the small molecule with which these proteins form complexes. An attempt to harmonize these seemingly divergent characteristics will be made in connection with a review of some well-known facts and a presentation of some new experimental aspects of non-specific protein interactions.

INTERACTIONS WITH ANIONS.—Anion complexes of serum albumin have been investigated most extensively. A selection of some typical binding data has been assembled in table 1.

TABLE 1.—FREE ENERGIES * OF BINDING OF SOME ANIONS BY SERUM ALBUMIN

Ion	ΔG_1^* cal/mole	reference
chloride	− 3660	1
thiocyanate	− 5460	1
acetate	− 3530	2
valerate	− 4510	2
caproate	− 4600	2
heptanoate	− 5270	2
caprylate	− 6100	2
octyl sulphate	− 6490	3
decyl sulphate	− 7660	3
dodecyl sulphate	− 8750	3
methyl orange	− 6410	4
azosulphathiazole	− 7150	4

* For purposes of comparison, ΔG_1 , the free energy of binding of the first ion taken up by albumin, has been listed.

It is apparent that a wide variety of anions can form complexes with albumin. Moreover these ions may compete with each other for attachment to this protein. Nevertheless, it should be emphasized that the sites of binding show some selectivity for particular structures. A small but detectable discrimination is apparent, for example, from competition studies of Karush,[5] who showed from variations in binding ability with concentration that one group of sites on albumin preferred dodecyl sulphate to certain azo dye anions while a second set of sites preferred the latter ions. More direct demonstrations of such preference have been presented in recent comparisons of interactions with isomers. Distinct differences have been observed in the affinity of serum albumin for positional isomers[6] in which substituents have been changed from an *ortho* to a *para* position on a benzene ring. Likewise, appreciable distinctions have been discovered in the attraction of albumin for stereochemical isomers.[7]

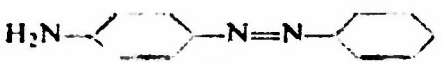
The effects of environmental changes, such as pH or temperature, on anion binding have been summarized in a recent review.[8] For the present purpose it seems desirable to draw attention primarily to the unanticipated increase in ability of serum albumin to bind anions at alkaline pH's. With increasing negative charge on the protein, a substantial decrease in ability to attract anions might be expected. Actually a slight increase in anion uptake is observed, which seems to be due to the uncovering of new sites on the protein molecule. Thus a change in

availability of tyrosine —OH groups has been demonstrated in human albumin.[9] Definite changes in configuration of the protein molecule occur at pH's above 7 and these seem to arise from a reversible swelling or unfolding produced by the strong internal electrostatic repulsions.[9]

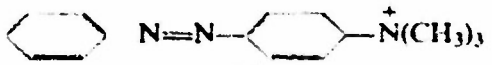
INTERACTIONS WITH CATIONS.—Recent studies indicate that protein interactions with cations show some similarities to those with anions as well as some major differences. Thus long-chain cationic detergents form complexes with serum albumin,[10, 11] egg albumin,[12] wool [13] and other proteins,[14-17] just as anionic detergents do. On the other hand, anions precipitate serum albumin at pH's slightly below the isoelectric point (i.e. as soon as the protein has a small net positive charge) whereas cations will not precipitate this protein until the pH is more than 3 units above the isoelectric point (i.e. only after the protein has acquired a substantial negative charge). Here we have an indication that with ions of equal size anions combine more strongly with albumin than do cations. Quantitative comparisons of binding ability [18] by the equilibrium dialysis method show unequivocally that such a conclusion is valid, not only for serum albumin but also for γ-globulin or trypsin.

We have reached similar conclusions recently in studies of albumin interactions with molecules comparable in size to the protein. Thus even at pH's above the isoelectric point anionic albumin forms complexes with anionic nucleic acids or polysaccharides.[19-21] On the other hand, our binding studies indicate that this protein is unable to combine significantly with cationic lysozyme even at pH 9 where albumin carries a negative charge [22] of about 30. Likewise Haurowitz [23] found that protamine does not precipitate native serum albumin at pH 7. It is apparent that anionic sites on albumin which should be suitable for binding of cations are blocked in some fashion. A suggestion as to the nature of this inaccessibility will be described shortly.

INTERACTIONS WITH NEUTRAL MOLECULES.—To augment the information available from studies with charged molecules, we have recently carried out a number of investigations of the binding of neutral organic molecules by albumin. Aminoazobenzene (I) was used as the typical small molecule since



(I)



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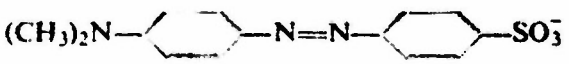


(III)

it is closely related to the cation (II) and anion (III) which have served as reference ions in earlier work.

Aminoazobenzene is definitely bound by bovine or by human serum albumin, as is evident from fig. 1. The affinity of albumin for this neutral molecule near pH 7 is somewhat less, however, than that for anionic methyl orange (III). Anions, furthermore, may compete with neutral molecules for common sites on the protein. Thus 0·1 M sodium thiocyanate reduces the uptake of aminoazobenzene significantly (fig. 1). On the other hand, sodium chloride does not produce an appreciable effect, a behaviour consistent with the much smaller affinity of albumin for this anion.

Since anions compete with aminoazobenzene, cationic groups of the protein can be assumed to be involved in interactions with neutral molecules. Corroboration of this view has been obtained from a study with acetylated albumin. This modified protein, in which cationic groups of lysine have been blocked, shows a

reduced affinity for aminoazobenzene (fig. 2). In contrast to studies with anions, the interpretation of this reduction in binding is not complicated by electrostatic effects due to the increased negative charge on the protein.

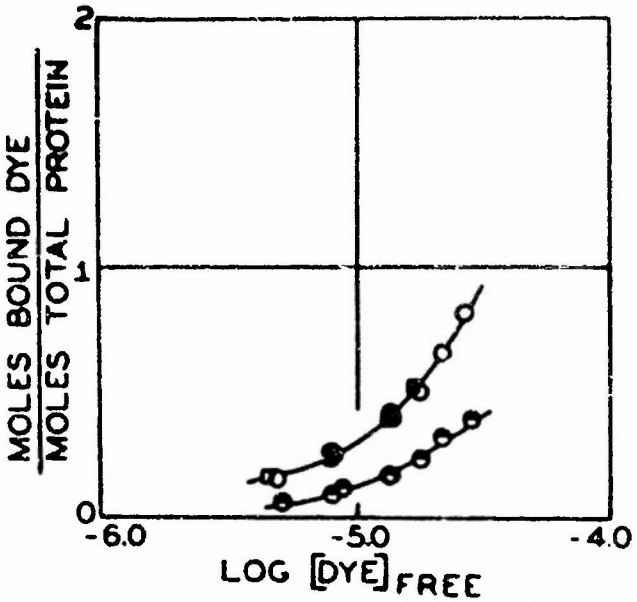


FIG. 1.—Binding of aminoazobenzene by bovine serum albumin at 25° C and pH 6·8; dye alone, ○; dye in presence of 0·1 M NaCl, ◐; dye in presence of 0·1 M NaSCN, ◒

More detailed investigations show that additional types of side chain are also involved in the binding of neutral molecules. First it has been found that 0·1 M

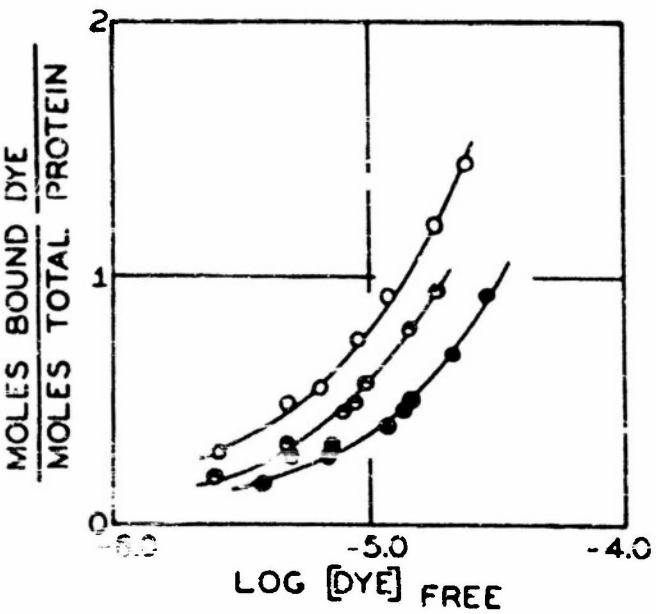


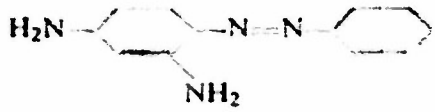
FIG. 2.

FIG. 2.—Binding of aminoazobenzene by proteins at 25° C and pH 9·2; bovine serum albumin, ○; acetylated bovine serum albumin, ◒; iodinated bovine serum albumin, ●. Protein concentration 0·2 % in each case.

thiocyanate ion displaces neutral aminoazobenzene much less readily than it does the anion methyl orange. More directly, it has also been observed (fig. 2) that

iodinated albumin, in which the diiodotyrosine phenolic group becomes ionized at pH 9, shows an even greater loss of affinity for neutral molecules than does acetylated albumin. Clearly tyrosine groups are also involved in the interactions of albumin with aminoazobenzene, the small molecule evidently acting as the acceptor end of a hydrogen bond.

It has been particularly interesting to find that the binding of neutral molecules by serum albumin increases substantially as the pH is raised. This trend can be seen over the pH range of 7 to 9 with aminoazobenzene in a comparison of the top curve in fig. 1 and fig. 2, respectively. More extensive studies over an even wider pH range (4 to 9) with chrysoidine (IV)



(IV)

give essentially the same results. At pH 9, chrysoidine is taken up by albumin with an affinity comparable to that of the anion methyl orange (III). As the pH is lowered, binding decreases until at pH 4 no significant complex formation is observed. (Complete abolition of binding at pH 4 may be facilitated in part by the conversion of chrysoidine to a singly- or doubly-charged cation at this acidity.)

This variation in binding of neutral molecules by serum albumin with change in pH offers an explanation for the seemingly divergent results reported by Karush [24] and by Klotz and Urquhart [25] in their investigations of the effect of pH on interactions with anions. In the former's experiments binding of methyl orange was found to decrease somewhat as the pH was increased from 7·5 to 9; in contrast the latter investigators found an increase over the same pH range. The aqueous anion-protein solution was equilibrated by Karush with an immiscible organic phase containing hexanol, which, as he pointed out,[24] competes with the anion. The present experiments show, furthermore, that neutral molecules are bound more strongly as the pH is increased. If neutral molecules and anions continue to compete for some common sites, it is clear that the anions should be more effectively displaced as the protein acquires a more negative charge. This increasingly effective competition of the neutral hexanol thus makes it appear that the binding of the anionic dye is decreasing. On the other hand, the experiments [25] in the absence of hexanol, suitably corrected [6] for buffer effects, show that binding of anions actually increases with increasing pH.

Thus the behaviour of serum albumin toward neutral molecules confirms the conclusions reached earlier in studies with anions that the configuration of this protein changes with pH and that new binding sites become available. With both anions and neutral molecules, furthermore, the loss in binding properties accompanying iodination of albumin [9] points clearly toward tyrosine groups being among the sites becoming accessible as the pH is increased.

For reasons described previously [9] it seems likely that the change in configuration of albumin with pH is not due to the dissociation of any specific side chains, such as histidine, but rather to a swelling or unfolding induced by the strong internal electrostatic repulsions developed in the protein molecule as its negative charge increases. Such internal repulsions would also be produced even at pH's only slightly above the isoelectric point as more and more anions are bound by albumin. It seems likely, therefore, that the very act of complex formation between albumin and anions makes more sites available for further binding. As has been suggested earlier,[26] such a mechanism could account for the continued failure of all attempts [8] to find an actual plateau in graphs of the number of ions bound against the free ion concentration.

COMPARISON OF PROTEINS.—In interactions with small anions serum albumin is far more effective than any other native protein. A small affinity for anions has been found with β-lactoglobulin. With relatively large anions, such as detergents, many other proteins also form complexes. In addition, at pH's acid to the isoelectric point, a few proteins such as ovalbumin also interact with small anions.

An explanation of the special affinity of albumin suggested in previous papers [26] places much stress on the relatively low content of hydroxy amino acids in this protein as compared to a non-binding protein such as γ-globulin. The basic assumption in this explanation is that —OH side chains form hydrogen bonds with other side chains containing $—COO^-$ or $\equiv NH^+$ groups. When the content of hydroxy amino acids is very high, cross links may be formed with practically all $—COO^-$ and $\equiv NH^+$ groups. A relatively rigid structure would thus be obtained in which $\equiv NH^+$ groups would be "bound" internally, hence they would be unavailable to small anions. On the other hand, in a protein such as albumin, fewer —OH groups are present and must be shared among a larger number of $—COO^-$ and $\equiv NH^+$ side chains. For reasons mentioned before,[26] it seems likely that an —OH . . . OOC— bond would be formed preferentially. The presence of fewer —OH groups would tend to make albumin a less rigid structure and in particular would permit many more $\equiv NH^+$ groups to remain in a relatively open position available for anion binding. Furthermore, being relatively unrestricted in their motion, cationic side chains could move into a position adjacent to a lipophilic residue and in this way make available a source of van der Waals' attraction in juxtaposition to the electrostatic centre.

In view of this emphasis on the role of hydroxy amino acids, it has seemed desirable to examine other proteins with few —OH side chains relative to anionic side chains. Recent analyses of α- and of β-casein [26a] indicate that these proteins fall in this category. It was therefore of interest to find that these proteins also bind methyl orange (fig. 3), although with an affinity substantially less than that of serum albumin.

The behaviour of albumin toward organic cations also fits the assumption that —OH groups interact preferentially with $—COO^-$ side chains. Because of this internal bonding an organic cation must have a much stronger interaction energy to be bound at a $—COO^-$ site than an organic anion requires to be bound at an $\equiv NH^+$ site. Among molecules of similar size and structure, the anions are therefore bound much more strongly than cations.

Complexes with neutral molecules seem to follow a pattern similar to that with anions in so far as comparisons among different proteins are concerned. Serum γ-globulin, for example, does not bind aminoazobenzene. Thus it seems again that the content of hydroxy amino acids is a critical factor, probably again because they make the protein molecule relatively rigid in configuration and hence unable to place suitable side chains in juxtaposition.

A most puzzling problem in connection with the unique properties of serum albumin that still remains essentially untouched is that of the effect of denaturation. It is not difficult to understand that prolonged exposure to alkali should abolish the binding ability of the protein, for the base hydrolyzes the polypeptide and hence separates the amino acid residues. The effect of heat, however, is perplexing. There is no doubt that exposure of albumin to temperatures in the neighbourhood of 100° C eliminates its affinity for anions.[27-29] Quite in contrast, however, heat generates binding properties in egg albumin, chymotrypsin and other proteins which show no binding in the native state.[29-33] Similarly, denaturation by alcohol destroys the affinity of serum albumin [28] whereas denaturation by acid produces binding ability in ovalbumin.[34]

With denatured ovalbumin at least, cationic side chains seem still to be involved in anion binding, for deamination reduces the affinity of the protein for methyl orange.[34] The effect of denaturation can thus be reconciled readily with current

molecular interpretations of the denaturation process. Presumably, the effect of heat, for example, is to break up the compact, rigid structure of ovalbumin and to give an extended open structure in which cationic sites are exposed and sufficiently mobile to be able to move into juxtaposition with a lipophilic residue and thereby provide the electrostatic and van der Waals forces to attract organic, as well as inorganic, ions. The unfolding action of the denaturant would also facilitate aggregation of molecules of ovalbumin, again because of exposed side chains.

Denaturation of serum albumin, on the other hand, clearly must involve some other mechanism. Major differences in the course of urea denaturation of serum albumin and of ovalbumin, as judged from changes in optical rotation and in viscosity, have also been observed by Kauzmann and his co-workers.[35] A possible explanation of these effects may lie again in the relatively low proportion of hydroxy amino acids in serum albumin. With relatively few internal —OH cross bonds, many side chains are free. Consequently the first effect of heat may be to produce aggregation of serum albumin molecules even in their folded state through side-

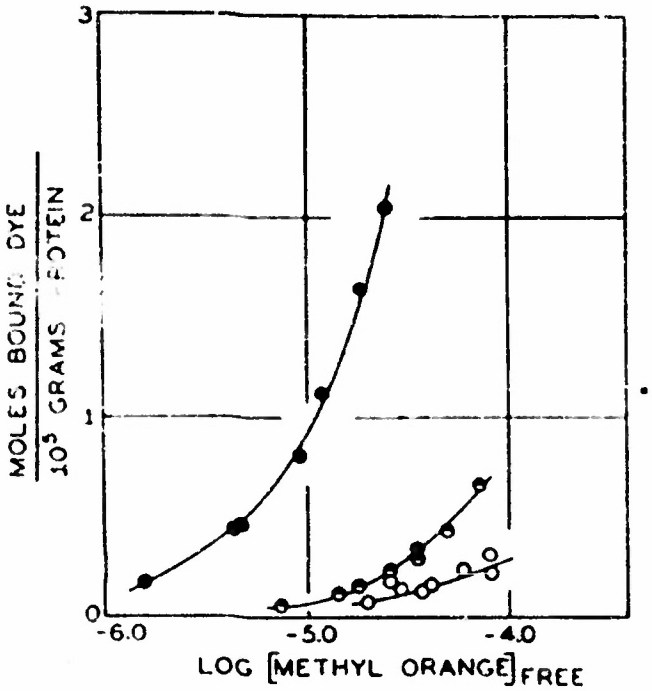


FIG. 3.—Binding of methyl orange by proteins at 0° C in phosphate buffer at pH 6·9; bovine serum albumin (0·2 %), ●; α-casein (1 %), ⊖; β-casein (1 %), ○.

chain interactions of separate molecules. In contrast, in rigid ovalbumin molecules with many internal —OH cross bonds such interactions could not occur until the protein molecule is at least partially unfolded to give access to side chains from other ovalbumin molecules. If serum albumin molecules can be aggregated even in their folded state, binding of anions should be decreased, for many sites that would normally be used binding would now be involved in inter-protein cross links.

This explanation of the difference in the effect of denaturation on binding can account also for the relative ease of reversal of denaturation of serum albumin as contrasted to ovalbumin.[35] With the former protein aggregates involve molecules which may still be folded, and hence under suitable conditions which permit disaggregation, at least some native folded molecules are released. In contrast, with ovalbumin disaggregation would release molecules which are still highly unfolded and disoriented and it would be highly unlikely that the original native configuration could be regained.

Thus a variety of aspects of non-specific protein interactions can be correlated in terms of a single assumption, that hydroxy amino acid side chains are the critical factor in determining the behaviour of the protein molecule toward small molecules. It seems worthwhile, therefore, to continue to use this picture as a basis for selection of proteins which may show non-specific affinity toward small molecules. It must be realized, nevertheless, that this viewpoint can explain only gross features of such interactions. As more precise information is obtained about molecules for which proteins such as serum albumin show preferential affinities, greater detail on configurational relationships among side chains will be obtained.

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